

# Identification and characterization of a pre-cleavage synaptic complex that is an early intermediate in Tn10 transposition

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**The Tn10 transposition reaction has been reconstituted *in vitro* on short linear substrate fragments encoding transposon ends. This permits the direct detection of protein–DNA complexes formed during transposition by gel retardation analysis. We demonstrate that a stable synaptic complex containing transposase and a pair of transposon ends forms rapidly and efficiently, prior and prerequisite to the double-strand cleavages involved in transposon excision. These observations extend the general analogies between the Tn10 and Mu transposition reactions, and also reveal significant differences between the two cases. The speed and simplicity of synaptic complex formation in the Tn10/IS10 reaction is suitable for a modular insertion sequence. In contrast, the relative slowness and complexity of this process in the Mu is necessary to permit transposition immunity and control of transposition by Mu repressor protein, two features specifically important for a temperate bacteriophage. Further dissection of the reaction leads to a tentative working model for events preceding the first double-strand cleavage.**

**Keywords:** IS10/protein–DNA complex/Tn10/transposition

## Introduction

Tn10 is a composite transposon consisting of a tetracycline resistance determinant flanked by inverted repeats of the insertion sequence IS10 (Kleckner, 1989; Kleckner *et al.*, 1995). IS10 itself consists of the coding region for the transposase protein plus essential terminal sequences (Figure 1). The two IS10 termini, referred to as 'inside' and 'outside' (or 'I' and 'O'), share a nearly perfect terminal inverted repeat of 23 bp (Halling and Kleckner, 1982). The outside end also encodes a specific binding site for Integration Host Factor (IHF) immediately internal to the terminal inverted repeat sequence (Huisman *et al.*, 1989; D.Morisato and N.Kleckner, unpublished observations).

Tn10 and IS10 transpose by a non-replicative mechanism (Bender and Kleckner, 1986; Haniford *et al.*, 1989; Benjamin and Kleckner, 1992); the element is excised from its donor site by a pair of flush double-strand cleavages and inserted into a target DNA site cleaved by staggered breaks 9 bp apart. The primary strand transfer product thus contains a symmetrical pair of 9 bp single-

strand gaps (Benjamin and Kleckner, 1989) that are subsequently repaired by host functions.

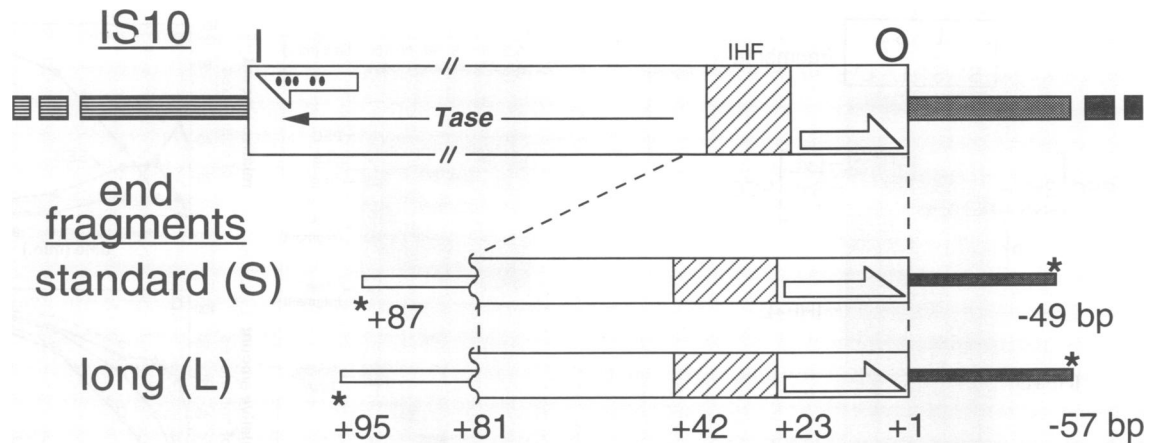
Integration into target DNA probably involves a single step nucleophilic attack as in the cases of Mu and HIV (Engelman *et al.*, 1991; Mizuuchi and Adzuma, 1991). IS10 transposase carries out all of chemical steps in the transposition reaction, cleavage and strand transfer (Benjamin and Kleckner, 1992; Chalmers and Kleckner, 1994). IHF serves as a regulatory element *in vivo* (Signon and Kleckner, 1995) and can play a positive accessory role under certain reaction conditions *in vitro* (below; Morisato and Kleckner, 1987; R.Chalmers and N.Kleckner, unpublished observations).

Analysis of the Tn10 transposition reaction *in vivo* has demonstrated that the chemical steps occur within a stable synaptic complex involving the two transposon ends and proteins, presumably including transposase. Stable complexes corresponding to the fully excised transposon segment and to a transposon segment that has undergone strand transfer have been identified (Haniford *et al.*, 1991). *In vivo* analysis has also demonstrated that interaction between ends is required as a prerequisite to double-strand cleavage at either end (Haniford and Kleckner, 1994).

The entire transposition reaction has been reconstructed *in vitro* using purified transposase and supercoiled substrates containing appropriately oriented pairs of Tn10/IS10 ends (Morisato and Kleckner, 1987; Benjamin and Kleckner, 1989; Chalmers and Kleckner, 1994). This analysis demonstrates that the transposon ends can identify a target DNA by random collision, target sequences may be located either within the transposon itself or on another molecule (H.Benjamin and N.Kleckner, unpublished observations; R.Chalmers and N.Kleckner, manuscript in preparation).

The results presented below further characterize the transposition reaction by analyzing the protein–DNA complexes formed by transposase and short linear fragments encoding transposon ends. This analysis has identified a stable synaptic complex that forms between pairs of transposon ends in the absence of cleavage. This pre-cleavage 'paired ends complex' (PEC) is capable of undergoing both cleavage and strand transfer. Additional observations suggest that the PEC is an obligatory precursor to the subsequent chemical steps. Also, kinetic analysis shows that cleavages at the two transposon ends within the PEC are temporally distinguishable, with very similar times observed for each cleavage step. The stability of the PEC is affected by both divalent metal ions and by mutations in the very terminal base pairs (bp 1–3) of the transposon end. These and other observations allow us to propose a working model for the pathway of the assembly of the synaptic complex.

Identification of a pre-cleavage synaptic complex extends the analogy between the IS10/Tn10 and Mu



**Fig. 1.** Transposon end substrate. Both the outside (O) and inside (I) ends of IS10 are transposition proficient and contain the 22 bp inverted repeat sequence; the inside end differs from the outside end at five positions (indicated by •). The substrate used in this work is a 136 bp fragment containing 81 bp of the outside end, which has an IHF binding site adjacent to the inverted repeat. The flanking DNA, consists of 34 bp of the *hisG1* hot spot (Halling and Kleckner, 1982). Additional base pairs originate from the cloning vector, pGC1. Radiolabel is indicated by \*. For the experiments described in Figure 3, the longer fragment, L, was generated by digesting the vector with different restriction enzymes which adds 8 bp of polylinker sequence to each end.

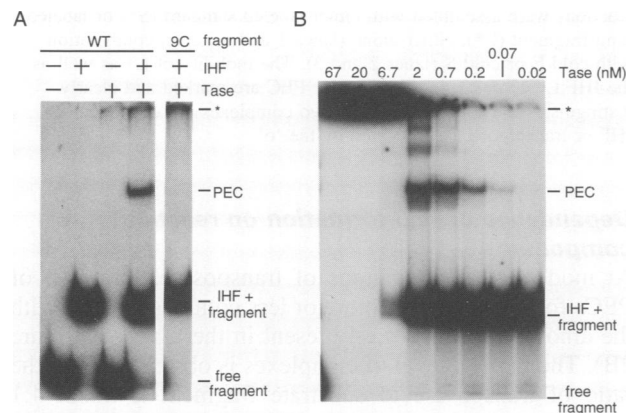
transposition reactions (Surette *et al.*, 1987; Haniford *et al.*, 1991; Mizuuchi *et al.*, 1992; below). However, the current analysis demonstrates that the formation of the pre-cleavage complex differs significantly in both speed and complexity in the two cases. These differences can be rationalized on the basis of differences in the biologies of the two elements.

## Results

### IS10 transposase forms a stable protein–DNA complex containing a pair of Tn10 ends

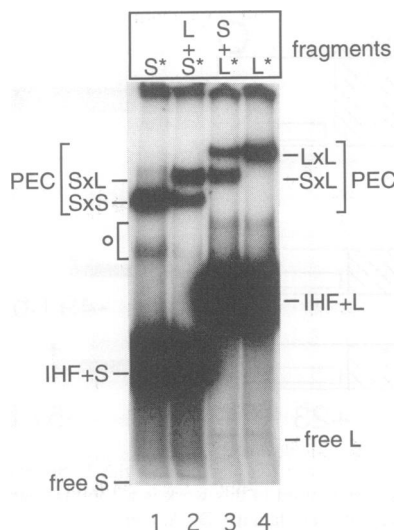
Incubation of purified IS10 transposase and IHF with a short DNA fragment containing the outside end of IS10 (the 136 bp S fragment, Figure 1) results in the formation of a stable protein–DNA complex that forms a discrete band in a polyacrylamide gel (Figure 2A, ‘PEC’). Formation of this complex is dependent upon the presence of transposase and upon the genetic integrity of the termini: no complex forms in the absence of transposase or if the substrate fragment carries a strong mutation within the region thought to participate most intimately in transposase binding (bp 9, Huisman *et al.*, 1989; Figure 2A, right). Under optimal conditions as much as 30% of substrate fragment is incorporated into the observed complex. The remaining fragment is mostly present bound to IHF; some is present in protein–DNA aggregates in the wells.

The observed transposase–DNA complex consists of a pair of transposon end substrate fragments held together by proteins (Figure 3). Complexes formed separately on substrate fragments differing by 16 bp in length (S and L; Figure 1) migrate with significantly different mobilities: in reactions containing either type of fragment alone, single discrete species of appropriate relative mobility are observed (Figure 3, outer two lanes). In reactions containing a mixture of the two fragments, however, three species are formed. In such reactions, if only one of the two substrate fragments is labeled, two species are observed, one characteristic of the labeled fragment alone and a second with a mobility intermediate between the



**Fig. 2.** Requirements for PEC formation. (A) Standard transposition reaction buffer conditions included IHF and transposase as indicated. The presence of both proteins is required for efficient PEC formation. When IHF is included, a majority of the fragment is present as a complex with IHF. When transposase is present, some of the fragment is retained in the wells (\*), presumably in complex protein–DNA aggregates. The 9C fragment is isogenic to the standard fragment except for a mutation at bp 9 in the transposon end, which decreases transposition >5000-fold *in vivo* when present at both ends of the transposon. (B) Standard reaction conditions included varying amounts of transposase as indicated above each lane. The ladder of bands with a mobility slower than the PEC are unassigned, but may be simple aggregates of the PEC. At higher amounts of transposase, all of the fragment is retained in the wells.

two complexes observed with either fragment alone. Moreover, this same intermediate mobility species is observed irrespective of which component substrate fragment is labeled (Figure 3, central lanes). We infer that the mixed reactions contain three species, one containing two S fragments, one containing two L fragments and a third of intermediate mobility containing one S and one L fragment. We therefore refer to this species as a ‘paired ends complex’ or ‘PEC’. No species corresponding to binding of transposase to a single terminus fragment is observed under these conditions.



**Fig. 3.** The PEC contains two transposon end fragments. Transposition reactions containing different length fragments were assayed to determine the DNA content of the complexes. The long fragment (L) is isogenic to the standard fragment (S) except that it contains an additional 16 bp of polylinker material. Standard transposition reactions were assembled with either labeled standard (S\*) or labeled long fragment (L\*), either alone (lanes 1 and 4), or in combination with cold L or cold S (lanes 2 and 3). The mobility of L\* as well as the IHF:L\* complex and the L\* $\times$ L\* PEC are marked and clearly distinguishable from those of S\*. Two complexes not dependent on IHF or transposase are indicated by the 'o'.

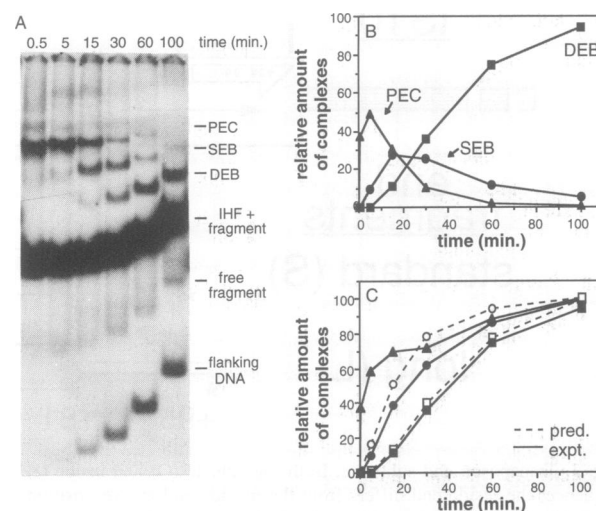
#### Dependence of PEC formation on reaction components

At moderate concentrations of transposase, the level of PECs formed increases more or less commensurately with the amount of transposase present in the reaction (Figure 2B). The highest level of complexes is observed when the ratio of transposase to substrate fragment is about 4:1 (~2 nM transposase; Figure 2B). In this situation, two lower mobility species are also observed which could represent aggregated PECs and/or other forms.

As the transposase concentration is increased above the optimal level, virtually all of the substrate fragment is found in aggregates that remain in the well of the gel. Very little fragment migrates at the positions of either the PEC or of fragment bound by IHF. The composition of these aggregates is unknown. Unlike PECs however, the aggregates are not competent to carry out subsequent steps of the reaction (below; data not shown). IS10 transposase is known to have a strong tendency to aggregate (Morisato and Kleckner, 1987; Benjamin and Kleckner, 1992; Chalmers and Kleckner, 1994).

Formation of the PEC is absolutely dependent upon the presence of IHF (Figure 2A). If IHF is omitted from the reaction, the level of PEC decreases more than 1000-fold. The IHF concentration that gives an optimal level of PECs corresponds approximately to the lowest concentration at which virtually all fragment is bound by IHF in reactions from which transposase is omitted (data not shown) suggesting that IHF acts stoichiometrically. For this reaction, specific interaction of IHF with its binding site is required; mutations in the IHF site abolish the reaction and HU cannot substitute for IHF (data not shown).

Standard reaction conditions are 25 mM Tris, pH 7.5,



**Fig. 4.** Kinetics of PEC, SEB and DEB formation. (A) A standard reaction supplemented with 4 mM  $MgCl_2$  was assembled omitting the transposase and pre-warmed to 30°C. The time course was initiated by the addition of transposase. Aliquots were withdrawn at the times indicated and loaded directly onto a gel under tension. The curve of the bands up the gel is indicative of the different electrophoresis times of the individual samples. Two cleavage complexes, the SEB, corresponding to cleavage of one of the two transposon ends, and the DEB, corresponding to cleavage at both ends, as well as the cleaved flanking DNA are visible, in addition to the bands observed in Figure 2. (B) The relative amounts of the complexes in the gel from (A) were quantified by phosphorimaging. Before plotting, the quantities were corrected for the relative amount of label expected in each complex. (C) Cumulative curves. Cumulative curves corresponding to the number of complexes which have entered a particular stage have been calculated for the PEC and the SEB (solid lines). The theoretical curves predict the exit from the PEC and SEB stages (broken lines); these calculations are detailed in Materials and methods. Symbols are as for (B).

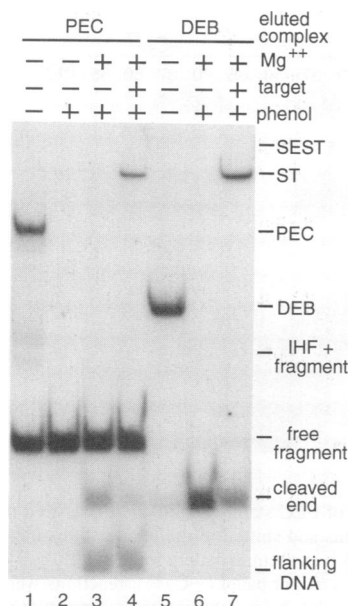
10 mM DTT, 19% glycerol, 100 mM KCl. Divalent metal ions, while not required for efficient formation of PECs, increase the stability of the complexes (see below).

#### The pre-cleavage PEC forms rapidly and is a precursor to complexes containing cleaved transposon end(s)

In reaction mixtures that contain  $Mg^{2+}$  in addition to the standard reaction components, addition of transposase results in formation of PECs which then undergo double-strand cleavages at both transposon ends within the complex (Figure 4).  $Mn^{2+}$  will also support cleavage, though less efficiently;  $Ca^{2+}$  does not support cleavage (data not shown).

In such a time course experiment, PECs appear rapidly. A prominent band is observed even in a time point taken as rapidly as possible after transposase addition. The PECs observed under these conditions have been formed in the reaction mixture, not during gel electrophoresis: although the addition of non-specific DNA to the reaction rapidly inhibits PEC formation, the addition of non-specific DNA at the time of assaying does not reduce the level of PEC observed (data not shown).

At subsequent times, two new complexes appear in succession. Within these complexes, either one substrate fragment has been cleaved at the end of the transposon ('single end break' or 'SEB') or both fragments have been



**Fig. 5.** Eluted PEC and DEB complexes are competent for cleavage and strand transfer. PEC and DEB complexes such as those in Figure 4 were eluted from a gel by excising the appropriate gel area and soaking in standard reaction buffer overnight. Eluted complexes were split into aliquots for further manipulation. Aliquots were either not supplemented (lanes 1, 2 and 5), or adjusted to 4 mM  $MgCl_2$  (lanes 3 and 6) or 4 mM  $MgCl_2$  and 200 nM (1  $\mu g$ ) target DNA plasmid (lanes 4 and 7) and incubated at room temperature for an additional 24 h. The reactions in lanes 2, 3, 4, 6 and 7 were subsequently phenol-chloroform extracted before loading. Possible strand transfer products are denoted either as ST, corresponding to the labeled linear strand transfer product which would arise from the transfer of both transposon ends to a single target DNA site or SEST, corresponding to labeled nicked circular target DNA which would arise from the transfer of a single transposon end to a target DNA.

cleaved ('double end break' or 'DEB'). Analysis of the DNA components of the two species as purified from a gel similar to that in Figure 4 demonstrates that the SEB complexes contain a 1:1 ratio of uncleaved substrate fragment and fragment cleaved at bp 1 (data not shown) and that the DEB complexes contain only the latter fragment (Figure 5, lane 6).

The flanking donor DNA segment, resulting from cleavage, migrates at the position expected for the corresponding free DNA fragment (Figure 4), suggesting that it is not stably bound within the synaptic complex after cleavage. It seems most likely that the shifts in the mobility of the synaptic complex observed upon cleavage at one or both ends results largely from successive loss of the flanking DNA component(s).

Additional experiments provide more evidence that the PEC, SEB and DEB complexes comprise a single linear reaction pathway, and that this pathway is the only route by which cleavages can occur. (i) The PEC is capable of giving rise directly to the two cleavage complexes. PEC complexes formed under standard reaction conditions and eluted from a gel similar to that in Figure 2A will undergo efficient cleavage at both component transposon ends if they are subsequently incubated with  $Mg^{2+}$  (Figure 5, lanes 1–3). (ii) Formation of the PEC not only precedes but also is required for cleavage. If PEC formation is blocked by the addition of non-specific DNA before addition of transposase, no detectable level of the fragment

diagnostic of cleavage is generated. More importantly, if PEC formation is blocked at a submaximal level by addition of non-specific DNA after some PEC has accumulated, the level of cleavage observed in a reaction is proportional to the level of PEC that has already formed (data not shown). (iii) There are no species detected under standard reaction conditions that might be manifestations of cleavage in the absence of synaptic complex formation. Such a process should yield either a corresponding protein–DNA complex, or, if such a complex is unstable, free fragment corresponding to the cleaved transposon end, neither of which are observed (Figure 4A).

### Detailed kinetics of the reaction pathway through DEB formation

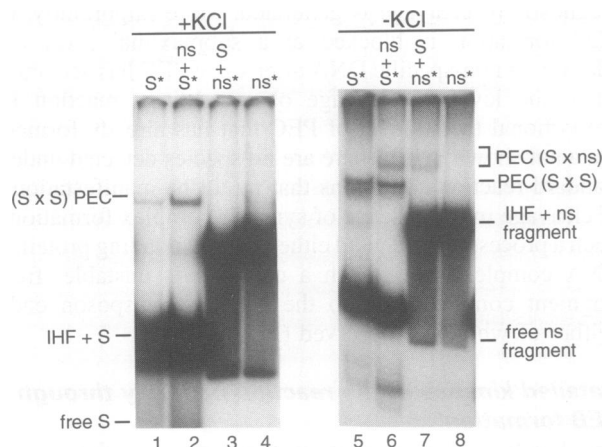
The kinetics of formation of the three complexes are revealed in more detail by quantitation of the abundance of the three species of interest as a function of time after transposase addition (Figure 4B and C). PECs are maximal at 5 min and decline thereafter; SEB complexes are observable at 5 min, maximal at ~20 min and decrease thereafter; DEB complexes accumulate progressively with time. The average time that a complex remains in each of the first two stages before it continues to the next can be estimated from the areas under the two corresponding curves (see Materials and methods). The average lifetime of the PEC is 12 min and the average lifetime of the SEB complex is 15 min.

The lifetimes of the PEC and SEB complexes can be used to calculate theoretical cumulative curves which predict the exit of complexes from the two stages (Figure 4C). This calculation assumes that the reaction is irreversible and that the PEC and SEB species are both kinetically homogeneous (see Materials and methods). The entry of complexes into the SEB and DEB species can be calculated from the observed data by summing, for each time point, the number of complexes present at the stage of interest or beyond. The calculated curve for exit from the PEC stage corresponds well to the experimental curve for entry into the SEB stage; similarly, the calculated SEB exit curve corresponds well to the experimental curve for entry into the DEB stage. Thus, these three observed species are likely the only long-lived complexes formed during the cleavage reaction.

### DEB complexes are competent for strand transfer

Strand transfer can be assayed by incubating complexes with a supercoiled target plasmid DNA. If both transposon ends attack the same target site, the plasmid will be linearized and will carry the two radiolabeled transposon end fragments at its ends. Gel-purified DEB complexes undergo strand transfer: the diagnostic linear species is observable after phenol–chloroform extraction of the DNA (Figure 5, lane 7; ST). Gel-purified PEC complexes incubated with  $Mg^{2+}$  and target DNA also give rise to strand transfer complexes, presumably after cleavage of both transposon ends (Figure 5, lane 4). Without elution, >90% of complexes undergo strand transfer. Of the eluted complexes, only 30–50% undergo strand transfer, presumably because the elution process has in some way compromised their ability to function.

Strand transfer of only one end within the DEB complex to target DNA would result in the production of a labeled



**Fig. 6.** Incorporation of non-specific DNA into PEC under permissive conditions. Mixed fragment experiments analogous to Figure 3 were assembled using the standard fragment and a non-specific fragment. Left panel: the standard fragment (S) or the non-specific fragment (ns) were labeled and their ability to form PEC complexes was assayed either when present alone (lanes 1 and 4), or in combination with cold S or cold ns (lanes 2 and 3). Right panel: lanes 5–8 are identical to lanes 1–4 except that the reactions omitted the 100 mM KCl usually present. One major and one minor complex are specific to the presence of the standard fragment and the non-specific fragment together.

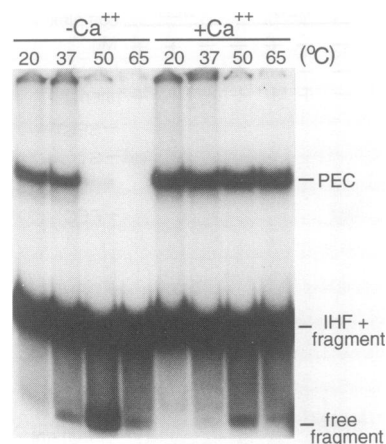
nicked circular species. Nicked circles generally represent <5% of the product and are not detectable in this experiment (Figure 5, SEST). There is no evidence for lower molecular weight species corresponding to the occurrence of multiple strand transfer events into a single target molecule. This is not unexpected given that target molecules are present in a 200-fold molar excess over synaptic complexes under these conditions.

#### **The kinetics of transposition on linear fragments are very similar to those observed in other assays**

The kinetics of cleavage observed in this assay are similar to those observed on supercoiled substrates. In reactions with supercoiled substrates, cleavage products are prominent after ~20 min both *in vivo* (Haniford *et al.*, 1991; Haniford and Kleckner, 1994) and *in vitro* (R.Chalmers and N.Kleckner, in preparation). The formation of a pre-cleavage complex must be rapid on supercoiled substrates *in vitro* as well, as the reaction is unaffected by addition of non-specific DNA after 10 min (data not shown). Similarly, in the linear fragment assay as in the other two situations, strand transfer is the slowest step in the reaction, requiring ~2 h from the time of initiation of the reaction (data not shown; Haniford *et al.*, 1991; R.Chalmers and N.Kleckner, in preparation).

#### **Formation of synaptic complexes on inside end and non-specific DNA substrate fragments**

On short fragments encoding the IS10 inside end sequence, complexes are formed only very inefficiently,  $\leq 1\%$  of total substrate, as compared with 30% for outside end substrate fragments (data not shown). Functional synaptic complexes do form efficiently, however, between an outside end fragment and an inside end fragment. The formation of these mixed complexes remains dependent upon the presence of IHF.



**Fig. 7.** Analysis of PEC stability. PEC were prepared under standard reaction conditions and shifted to the temperatures indicated above each lane for 1.5 h, and immediately loaded. Left half ( $-Ca^{2+}$ ): standard reactions. Right panel ( $+Ca^{2+}$ ): reactions were supplemented with 4 mM  $CaCl_2$  1 h before shifting the temperature of incubation.

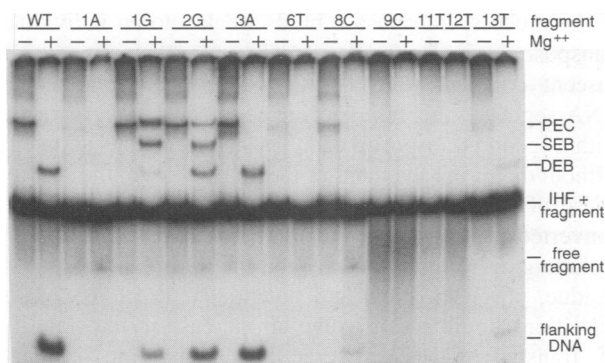
An outside end fragment is also capable of organizing a synaptic complex in combination with non-specific DNA, but only under special permissive reaction conditions, and the resulting complexes are destabilized by the addition of a divalent cation (Figure 6). Specifically, synaptic complexes form between an outside end substrate fragment and a non-specific DNA fragment in reaction mixtures containing 23 mM NaCl, but not under standard conditions, containing in addition, 100 mM KCl. Furthermore, when  $Mg^{2+}$  is added to a reaction mixture containing such complexes and then analyzed by gel retardation, no complexes are seen, suggesting that they have been partially or fully dissociated; also, no cleavage is observed (data not shown).

#### **Stability of complexes and effects of divalent cations on stability**

$Ca^{2+}$  stabilizes pre-cleavage complexes under standard reaction conditions. In the absence of divalent metal, the PEC is stable to incubation for 1.5 h at 37°C but is partially or fully disrupted by similar incubation at 55°C or 65°C respectively (Figure 7, left half). In the presence of  $Ca^{2+}$ , PECs survive all of these treatments. Synaptic complexes formed in the absence of divalent metal ion can also be stabilized by the absence of DNA beyond bp 1. Complexes formed under standard conditions on 'pre-cleaved' transposon end fragments (fragments lacking flanking DNA) are more heat resistant than standard PEC complexes (data not shown).

#### **Effects of mutations in the inverted repeat**

Within the terminal inverted repeat at the outside end of IS10, two important regions of sequence have been identified (Huisman *et al.*, 1989; Haniford and Kleckner, 1994). The primary site for interaction of transposase appears to be bp 6–13. The most terminal base pairs, bp 1–3, in contrast, are known to affect steps at or after interaction between the ends and are proposed to be involved primarily in such steps (Huisman *et al.*, 1989; Haniford and Kleckner, 1994; Kleckner *et al.*, 1995). More specifically, mutations 1A, 1G, 2G and 3A all exhibit defects in the transition



**Fig. 8.** Effect of mutations in the inverted repeat on PEC formation and cleavage. Standard reactions were assembled using substrates isogenic to the wild-type substrate with the exception of the mutation indicated. Reactions were supplemented with 4 mM MgCl<sub>2</sub> as indicated. Mg<sup>2+</sup> was added after 2 h; incubation of the reactions was continued at room temperature overnight.

from cleavage to strand transfer. Furthermore, mutations at bp 1 and bp 2 also exhibit defects after ends interaction and before cleavage at the mutant end (Haniford and Kleckner, 1994).

The short linear fragment assay has been used to examine the effects of mutations within the terminal inverted repeat on formation and stability of synaptic complexes (Figure 8). The observed patterns of effects correspond well with those predicted from previous analyses. Mutations in bp 6–13 all quantitatively decrease complex formation, with the same hierarchy as observed for the transposition defects *in vivo*. After a 24 h incubation, fragments bearing the 9C, 11T or 12T mutation (>25 000-fold transposition defect *in vivo*) yield no detectable complexes, fragments bearing the 6T or 13T mutation (~3000-fold transposition defect) yield barely detectable levels of complexes, and a fragment bearing the 8C mutation (300-fold transposition defect) yields a significant amount, but less than wild-type level, of complexes. In contrast, three of the four terminal base pair mutations (1G, 2G and 3A), whose *in vivo* defects range from 14-fold to 1000-fold, have essentially no effect on the level of synaptic complex formed under standard conditions. However, the fourth mutation, 1A, which has a 1000-fold transposition defect *in vivo*, causes a severe defect in synaptic complex formation.

The PECs formed on mutant end fragments can be subdivided into four different categories according to their behavior upon addition of Mg<sup>2+</sup> (Figure 8 and data not shown). Class 1: the low levels of complexes formed on 1A, 6T and 8C ends are destabilized by the presence of either Mg<sup>2+</sup> or Ca<sup>2+</sup>, but when cleavage does occur, it occurs at both transposon ends. Class 2: the low levels of complexes formed on 13T ends are stabilized by the presence of either Ca<sup>2+</sup> or Mg<sup>2+</sup> and undergo cleavage at both ends in the presence of Mg<sup>2+</sup>. Class 3: complexes formed on 1G mutant ends undergo aberrant cleavage. When analyzed by gel retardation after a 3 h incubation with Mg<sup>2+</sup>, approximately half of the complexes migrate as PECs and approximately half migrate as SEBs in a gel. Significant amounts of DEBs are observed only at very late times, usually after incubation for several days. Class 4: complexes formed on the 2G and 3A mutant end undergo cleavage more slowly than wild-type; additionally,

the cleaved complexes formed on the 3A end appear to be slightly unstable because protein-free cleaved transposon end fragment appears in the mutant reaction, but not in a wild-type reaction, after very long incubation periods.

## Discussion

The observations presented above demonstrate that IS10 transposase, in conjunction with IHF, has the capacity to organize a stable synaptic complex between a pair of Tn10 ends before, and as a prerequisite for, occurrence of any of the chemical steps of transposition. These complexes are therefore functionally analogous to Mu pre-cleavage complexes (Type O or SSC; Mizuuchi *et al.*, 1992). Analogous complexes are inferred to exist in other transposition reactions as well (e.g. Tn7, Bainton *et al.*, 1993).

### Relationship of IS10/Tn10 synaptic complex formation to that of bacteriophage Mu

The observations presented above extend the general analogies between the IS10/Tn10 transposition reaction and that of bacteriophage Mu. Like Mu, Tn10 transposition involves the formation of a synaptic complex before any of the chemical steps, which then occur in the context of this synaptic complex (Surette *et al.*, 1987; Haniford *et al.*, 1991). As the reaction progresses, the synaptic complex undergoes a progressive stabilization, with each form more stable than the preceding form (Surette *et al.*, 1987; Haniford *et al.*, 1991; Mizuuchi *et al.*, 1992; above).

The Tn10 and Mu reactions are, however, significantly different. A number of these distinctions likely reflect the different biological role of transposition in the lifestyles of the two elements. Most notably, assembly of the Mu pre-cleavage complex is very slow (Mizuuchi *et al.*, 1992) whereas assembly of the Tn10 complex is very rapid. In addition, perhaps correspondingly, the structural requirements for assembly of the Mu complex are much more elaborate than for Tn10. The Tn10 reaction requires only one transposase binding site at each terminus. Assembly of the Mu complex requires multiple binding sites at the transposon ends, plus a third determinant, the IAS 'enhancer' sequence, which is located some distance away from either end and contains additional transposase binding sites (Leung *et al.*, 1989; Mizuuchi and Mizuuchi, 1989; Surette and Chaconas, 1992). Also, efficient transposition of intact Mu bacteriophage *in vivo* requires a centrally located DNA gyrase binding site (Pato *et al.*, 1990). Mu is a bacteriophage of 39 kb in length (Howe, 1987); such an element can easily accommodate extra determinants. An insertion sequence of 1.3 kb, in contrast, must be a compact functional module which utilizes a minimum of determinants.

In particular for Mu, the participation of a third element, the IAS, permits the reaction to be controlled directly by Mu repressor protein (Mizuuchi and Mizuuchi, 1989; Mizuuchi *et al.*, 1992). Binding of Mu repressor at the IAS blocks binding of transposase at that site. This feature, which is analogous to direct inhibition of DNA replication in the case of phage  $\lambda$  (Dove *et al.*, 1971), is part of the process that commits Mu to undergo lysogeny rather than a lytic growth cycle. No analogous critical decision point requiring additional modulation exists for Tn10, although

the state of the host replication influences the frequency of Tn10 promoted events (Roberts *et al.*, 1985). In addition, Mu also regulates the choice of ends via the IAS (Baker and Mizuuchi, 1992). For IS10/Tn10, such a constraint does not apply; rather a diversity in the choice of ends likely produces a variety of evolutionarily significant events (Kleckner *et al.*, 1995).

Assembly of the Mu synaptic complex must be slow in order to permit time for the establishment of 'target immunity', an independent set of reactions required to prevent insertion of Mu into itself and destruction of the intact phage (Adzuma and Mizuuchi, 1988; Mizuuchi *et al.*, 1992). Tn10/IS10 transposition has no such requirement and assembly is rapid. For an insertion sequence like IS10, which can form composite transposons, intratransposon events are advantageous rather than deleterious because they generate new composite transposable elements and thus promote dissemination of the component IS element (Kleckner *et al.*, 1995). Correspondingly, IS10 does not exhibit target immunity; moreover, intratransposon events are differentially promoted by host factors (Signon and Kleckner, 1995).

#### **Formation and stability of IS10/Tn10 pre-cleavage synaptic complexes**

Stable paired ends complexes form very rapidly under standard conditions, which do not include a divalent metal ion. Under these conditions, we have not observed any species that might correspond to intermediates in PEC formation. Intermediates might either be unstable and/or too transient to have been detected. Potential intermediates are detected, however, under very low salt reaction conditions. PEC formation in this situation is slower than usual; moreover, at early times, a ladder of bands is observed which ultimately disappears and is replaced by the PEC (data not shown). The nature of these species is not known, but they could represent transient binding of transposase to individual DNA fragments. Also under such conditions, an outside end can make a stable complex with DNA fragments devoid of transposon end sequences (above).

Formation of a PEC is not critically dependent upon determinants that define the catalytic active site of the synaptic complex. Most base pair changes at the three terminal positions (bp 1–3) have little effect on PEC formation. In addition, divalent metal ions, which are required for cleavage and strand transfer, are not required for PEC formation.

It is clear, however, that both of these determinants are relevant to PEC integrity before cleavage. One mutation at the terminal base pair (1A) greatly reduces the level of pre-cleavage complexes observed in the absence of divalent metal, suggesting that an inappropriate base pair at this position can be disruptive. Moreover, divalent metal ions can either stabilize or destabilize paired ends complexes formed under standard conditions with wild-type or diverse non-wild-type substrates. These observations are compatible with the hypothesis developed for Mu that the active site of the transposase protein participates directly in formation of pre-cleavage synaptic complexes (Haniford and Chaconas, 1992; Baker *et al.*, 1993).

The observations above can be ordered into the following working model for PEC formation. In the first phase

of the process, transposase binds weakly to an individual transposon end. Bound transposase can then organize a nascent complex together with a second, non-specific DNA segment; this non-specific segment is then replaced with a second transposon end. These interactions all occur without direct involvement of the active site. In the second phase of the process, this initial paired ends complex is converted to a form in which the active site of the protein, including divalent metal ions and the terminal transposon residue, plays a critical and potentially direct role. This transition should also involve an increase in the stability of transposase–DNA interactions along the primary recognition region. This second phase might correspond to a transition that serves to bring transposon termini and flanking DNA and the protein active site into juxtaposition for cleavage. It is expected that this event should impose additional requirements on the synaptic complex. Since PECs formed on pre-cleaved ends are more stable than those formed on intact substrate fragments, it can be inferred that flanking DNA is intrinsically destabilizing to a PEC formed in the absence of divalent metal. Flanking DNA destabilization is also observed for Mu transposition (Craigie and Mizuuchi, 1987).

The only other factor that plays an important role in PEC formation in this assay is IHF. IHF is not required, however, under optimal conditions for *in vitro* transposition of outside end transposons present on supercoiled plasmid DNA substrates, although even under these conditions IHF can increase the level of the reaction (R.Chalmers and N.Kleckner, unpublished observations). IHF is not required for intermolecular transposition of Tn10 *in vivo* (Signon and Kleckner, 1995). IHF may be required to compensate for the absence of supercoiling or some other disparity between the three reaction conditions.

We do not detect a stable species corresponding to the binding of the transposase to a single end regardless of whether transposase is intact (above), or present as proteolytic fragments (D.Kwon and N.Kleckner, in preparation) or assayed for by DNase I footprinting (D.Morisato and N.Kleckner, unpublished). This is unlike the observation for several other transposition systems characterized *in vitro* [e.g. IS903 (Derbyshire and Grindley, 1992), IS50 (Wiegand and Reznikoff, 1994), IS1 (Escoubas *et al.*, 1991), Mu (Craigie *et al.*, 1984),  $\gamma\delta$  (Wiater and Grindley, 1991)]. Single end binding may be simply more stable in the other transposition systems; specifically for Mu and  $\gamma\delta$ , stable single site binding can function in regulatory processes besides transposition *per se* (e.g. target immunity; Goto *et al.*, 1987; Darzins *et al.*, 1988). For the IS elements listed above, however, cleavage and strand transfer activities have not been demonstrated *in vitro*. In an extreme view, these transposases may be preferentially stabilized in a binding proficient, catalytically inert conformation, and perhaps, under *in vitro* conditions in which transposition occurs, stable single site binding may be undetectable for these elements as well. Stable specific single end binding is also not observed for several retroviral integrase proteins (Roth *et al.*, 1988; van Gent *et al.*, 1991) for which cleavage and strand transfer activities have been demonstrated *in vitro* (Bushman *et al.*, 1990; Craigie *et al.*, 1990).

### Cleavage at the transposon ends

These experiments show that double-strand cleavage of the first and second transposon ends are temporally separable events; stable protein–DNA intermediates corresponding to both cleavage species are observed. Temporal separation of the two cleavage events has also been observed for another transposon, Tn7 (Bainton *et al.*, 1991) as well as for Tn10 itself *in vivo* (Haniford and Kleckner, 1994).

Detailed kinetic analysis shows that cleavage of the first end occurs about 12 min after PEC assembly and cleavage of the second end occurs on average about 15 min after the first. Since each double-strand cleavage requires cleavage on both strands of the duplex, these kinetics are governed by the rate-limiting step required for the second strand cleavage at each end.

Are events at the two transposon ends mechanistically coupled? It is unlikely that events at the first end promote or accelerate events at the second end, since the time which passes between the first and second cleavage is, if anything, longer than that required for the appearance of the first cleavage. However, it remains possible that the events at one end can influence the cleavage at the second end via the synaptic complex. Cleavage kinetics of complexes formed on the 1G mutant end are biphasic; qualitatively similar aberrant kinetics are obtained when  $Mn^{2+}$  is substituted for  $Mg^{2+}$  in the wild-type reaction (data not shown). Thus, these two changes, which affect likely components of the active site, impose a block to the cleavage reaction. The aberrant cleavage kinetics observed could be dependent on communication between the ends within the synaptic complex, for example if aberrant timing of the cleavages at the two ends is responsible for the biphasic nature. These kinetics might also arise via an independent leaky block to cleavage at each end, with the asymmetric distribution of cleavage products being a trivial consequence of having two ends in a single complex.

## Materials and methods

### Transposition reactions

**DNA components.** The standard transposon end substrate fragment (Figure 1, 'S') contains bp 1–81 from the outside end of IS10, 34 bp of sequence next to bp 1 which are identical to those found adjacent to IS10-Right at a particular hot spot *hisG1* (Kleckner *et al.*, 1979; Halling and Kleckner, 1982), and 21 bp of additional sequence derived from the polylinker of the pGC1 cloning vector (6 bp flanking the transposon end and 15 bp adjacent to the *hisG1* DNA; Myers *et al.*, 1985). This fragment was generated by a *Bgl*II and *Sal*I double digest of plasmid pNK1935 (Huisman *et al.*, 1989). The longer transposon end substrate fragment (Figure 1, 'L') was generated by cleavage of the same plasmid at other sites flanking the IS10 sequences, *Eco*RI and *Cl*aI. Substrate fragments containing a mutation in the inverted repeat sequence were generated by *Bgl*II/*Sal*I cleavage of plasmids isogenic to pNK1935 (Huisman *et al.*, 1989): pNK2551 (1G); pNK2552 (1A); pNK2553 (2G); pNK2554 (3A); pNK1907 (6T); pNK2556 (8C); pNK1908 (9C); pNK1910 (11T); pNK2557 (12T); pNK1911 (13T). The non-specific DNA fragment used in Figure 6 is the 232 bp fragment of the *hisG1* hot spot, specifically, a *Bam*HI–*Bgl*II fragment from pNK2041 (constructed by D.Ahmamn).

Fragments were isolated and purified from restriction digested DNA by electrophoresis through a 2.5% NuSieve (FMC Corp.) agarose gel and purification from the gel slice with glass milk (Bio101). Purified fragment was then labeled at both ends by filling in with [ $\alpha$ - $^{32}$ P]dATP (NEN, 6000 Ci/mmol) using AMV reverse transcriptase (Promega). Labeled end fragment was separated from the unincorporated nucleotides by another glass milk purification step.

**Proteins.** Transposase was purified essentially as described in Chalmers

and Kleckner (1994). Immediately before its addition to the reaction, transposase was activated by dilution into 50% glycerol, 0.5 M NaCl, 10 mM DTT, 20 mM Tris, pH 7.5, 2 mM EDTA, 1 mg/ml BSA (R.Chalmers, unpublished). IHF was purified and kindly provided by P.Errada according to the method described in Nash *et al.* (1987). Protein concentrations were measured by Bicinchoninic (BCA) assay (Pierce).

**Reaction conditions.** Standard reaction conditions for formation of pre-cleavage (PEC) complexes were 22 mM Tris, pH 7.5, 1 mM TES, pH 7.5, 11.5 mM DTT, 19% glycerol, 23 mM NaCl, 100 mM KCl, 0.05 mg/ml BSA, 0.05 mM EDTA. Divalent cations were added in some experiments as indicated. Standard reactions contained ~10 fmol of transposon end fragment (~100 000 c.p.m.), ~40 fmol of transposase, and ~80 fmol of IHF in a total volume of 20  $\mu$ l. In all experiments, DNA and buffer were added to the reaction tube first; IHF was added next if desired; transposase was always added after DNA and IHF. Reactions were assembled and incubated at room temperature (approximately 20°C) for 3 h; similar results are obtained when the incubation period is extended to overnight, as for Figures 3, 5 and 6. For Figure 8,  $MgCl_2$  was added as required after a 2 h incubation, and the incubation for all reactions was continued overnight. For analysis, reactions were loaded directly onto a native 5% 29:1 acrylamide:bisacrylamide gel poured and run in 1× TAE; electrophoresis was at 200 V for 1 h. After electrophoresis, the gel was dried down on Whatman 3MM paper at 80°C for >40 min. Complexes were visualized by exposure of the gel to X-ray film (Kodak X-Omat) for 2 h or more as required.

For analysis of strand cleavage, the standard reaction buffer was adjusted to 4 mM  $MgCl_2$ . Reaction products were either assayed as protein–DNA complexes as described above or as free DNA species following removal of proteins with phenol:chloroform: isoamyl alcohol (25:24:1).

For analysis of strand transfer, ~250 fmol of supercoiled plasmid DNA (pNK2704; Bender and Kleckner, 1992) was added to a standard reaction mixture after PEC formation. The  $Mg^{2+}$  required for strand transfer was either present before addition of target DNA or was added after target DNA addition as appropriate. Strand transfer was detected as linearization of the substrate plasmid (see text). For such assays, proteins were removed by extracting the reaction with phenol–chloroform (1 vol). Similar results were obtained if the reactions were treated with proteinase K–SDS. DNA samples were then subjected to polyacrylamide gel electrophoresis as described above.

**Quantification.** For the cleavage time course in Figure 4A, the relative abundance of the various species was determined by quantification of counts in the corresponding DNA components using a Fuji Bas2000 Phosphorimager. For each species, the absolute number of counts was corrected for differences in the number of labeled ends and the differing efficiencies with which those labels were introduced in the labeling reaction.

### Elution of complexes from gels

To obtain complexes purified away from other reaction components, gel slices containing complexes were identified by autoradiography and excised. Each gel slice containing two reactions was mixed with 50  $\mu$ l standard reaction buffer and incubated at room temperature for at least 24 h during which time complexes diffused out of the gel and into the buffer; this procedure was scaled up by adding integral multiples of gel slices and reaction buffer as necessary. Approximately 50% of labeled DNA is present in the buffer phase after elution, of which 30–80% are still in intact complexes, with the remainder present as free DNA. Cleavage and strand transfer reactions were carried out with such complexes as described above except that the final concentration of intact complexes in the eluates was usually about one-fifth the concentration of complexes in the standard complete reaction.

### Kinetic analysis of cleavage

The observed physical complexes correspond to double-strand cleavage at first one end and then the other end within each given complex. The lifetime of each observed species can be determined by calculating the area under the curve for that state (sum over total time = relative number of complexes per unit time × unit time) and dividing that area by the total number of complexes (Padmore *et al.*, 1991). For the time course in Figure 4, the average lifetime of the PEC is 12 min and the average lifetime of the SEB complex is 15 min. These average lifetimes are similar to those measured when the PEC is accumulated before initiation of the time course with  $Mg^{2+}$ , which in experiment were measured at 11 min for the PEC and 13 min for the SEB.

This allows us to model the cleavage reaction as a simple irreversible

reaction of which the PEC is the initial substrate. Under this model, the lifetime of each complex is equal to the inverse of the rate constant for the transition out of the corresponding state. If each of the three observed physical complexes is an obligatory, kinetically homogeneous state, the simplest reaction is modeled as  $A \rightarrow B \rightarrow C$  with the rate constants  $k$  and  $k_2$ . The area under the curve that describes the appearance and disappearance of A is  $\int A(t)$  evaluated from 0 to  $\infty$ ; this would be  $\int A_0 e^{-kt}$  evaluated from 0 to  $\infty$ , which equals  $A_0/k$ . This relationship holds for any step in a series of single component, irreversible transitions. Thus, according to these assumptions, the rate constants for the transitions from PEC to SEB and from SEB to DEB, would be  $0.1 \text{ min}^{-1}$  and  $0.07 \text{ min}^{-1}$  respectively.

From these two rate constants it is possible to calculate cumulative curves that represent the fraction of total complexes that have proceeded beyond any particular stage, that is to the PEC complex and beyond and to the SEB complex and beyond. The two cumulative curves can be calculated as follows. A predicted 'exit from SEB' cumulative curve is given by  $\delta(\text{exit})/\delta t = k_2 \text{SEB}$ . Integration gives 'total complexes which have exited the SEB stage' as a function of time,  $t = k_2 \int \text{SEB}(t)$  evaluated from 0 to  $t$ , which is equal to  $k_2 \times$  (area under the SEB curve from 0 to  $t$ ). The correspondence between the calculated exit from SEB and experimental DEB appearance cumulative curves is reasonably good. A similar analysis can be performed for a cumulative SEB curve. A cumulative curve representing complexes which have gone as far as the SEB stage can be calculated as  $\text{SEB}_T = \text{SEB} + \text{DEB}$ , which can be compared with a predicted curve of  $k \times$  (area under the PEC curve from 0 to  $t$ ).

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